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Method Article

A Melanin bleaching method to prevent non-specific immunostaining of chicken feathers

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A B S T R A C T

Melanin in pigmented organs like the skin is known to react with 3,3′-diaminobenzidine (DAB) to give a brown colour indistinguishable from the colour that DAB imparts to target antibodies bound to specific antigens. This can lead to false positives in chicken feathers during immunoperoxidase staining. Here, we present a simple, fast and practical method for bleaching chicken feathers which can be applied prior to immunohistochemistry staining without affecting specific antigen-antibody binding. To our knowledge, this is the first report of a melanin-bleaching technique prior to immunoperoxidase staining techniques of chicken feathers for detection of pathogens. Optimisations of the method include:

- Removal of melanin from tissue sections using a short incubation with potassium permanganate followed by incubation with oxalic acid prior to immunostaining for improved specificity.
- This technique did not affect the antigenicity of infectious laryngotracheitis virus antigen and did not cause damage or detachment of tissues from the slides.

A R T I C L E I N F O
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Keywords: Feather follicles, Chicken, Diamminobenzidine, Melanin, Potassium permanganate, Oxalic acid
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Name and reference of original method:	*McGovern, J. and Crocker, J. (1987). The effects of melanin pigment removal on the peroxidase-antiperoxidase immunoperoxidase technic, Am. J. Clin. Pathol. 88, 480–3. Morris, D.J., Turgut, E., Thompson, K.D. (2002). The use of melanin bleaching for the immunohistochemical detection of Renibacterium salmoninarum. Bulletin-European Ass. Fish Pathologists 22, 33–36.*
Resource availability:	*All consumables and equipment required are described in the manuscript*

Method details

Feathers have been used to detect the nucleic acids of several pathogenic viruses of chickens including infectious laryngotracheitis virus (ILTV), Marek's disease virus, and fowlpox virus [2–5] as feathers are easy to sample and can be collected from live birds without significant welfare issues. Feathers have also been used to detect antigens of Marek's disease virus, feather and beak disease virus and avian influenza virus [10,16,20] by immunohistochemistry (IHC). The epidermis which lines the feather follicle is considered to have a poor host immune response against viral replication [7] and the dry keratinocytes that cover feather shafts may preserve viruses. However, feathers, particularly from coloured breeds, are rich in melanin granules [6] and the presence of melanin in tissues could result in false positive results or could masque the antigen-antibody interaction when 3,3′-diaminobenzidine (DAB) is used for staining [19]. DAB is used routinely as a chromogen for immunohistochemical staining, as it is cost effective and produces intense, insoluble, stable deposits that remain indefinitely. However, interpretation of findings using DAB as a chromogen can be hampered by the presence of melanin in tissues, which also imparts a brown colouring, nearly indistinguishable from the DAB-antigen complex. Previous studies [10,11] did not take into account the effect of melanin on IHC in feathers when using DAB.

In clinical immunohistochemical procedures, melanin bleaching using potassium permanganate and oxalic acid is used to remove melanin from heavily melanocytic tumours in canines, equines and humans [12,15,22]. Melanin bleaching techniques are also used for the removal of melanin from non-malignant tissues with high melanin contents such as the kidney in fishes and the skin in seals [14,21]. However, to our knowledge, this technique has not been reported for the detection of viral pathogens in poultry feathers when used in conjunction with IHC. Here we present a technique for melanin bleaching of chicken feathers prior to IHC staining for ILTV. Although ILTV DNA has been reported in several organs, there is little to no evidence of a viraemic phase during ILTV acute infection or of active viral replication outside of the respiratory tract and conjunctiva [9]. Therefore, the origin of ILTV DNA in chicken feathers and the potential of feather dander to transmit ILTV is unknown. This melanin-bleaching method was developed as a research tool to assess whether ILTV antigen could be detected in specific cells on feathers from ILTV DNA positive chickens.

Required reagents and consumables

- Xylene (Fronine Pvt. Ltd, Riverstone NSW, Australia)
- Absolute ethanol (Chem Supply, Gillman SA, Australia)
- Modified citrate buffer solution (Dako Target Retrieval 10x, S1699, Dako, USA): diluted in 1:10 ratio in PBS
- Trizmabase (Sigma-Aldrich, St. Louis, USA)
- Phosphate buffer solution (PBS) powder 0.01 M (Sigma-Aldrich)
- Equine serum (Sigma Aldrich)
- Triton (Sigma-Aldrich)
- Nickel (II) sulphate hexahydrate (Sigma-Aldrich)
- Diaminobenzidine (DAB) (Sigma-Aldrich)

- Rabbit anti-ILTV glycoprotein E Polyclonal Antibody (Cat. No. BS-1674R, Bioss, Woburn, USA)
- Anti-rabbit HRP (Cat. No. ASO9602 Agrisera, Vannas, Sweden)
- Harris hematoxylin (Sigma-Aldrich)
- Dibutylphthalate Polystyerene Xylene (DPX) (Sigma-Aldrich)
- Hydrogen peroxide
- PAP pen (Cat. No., 2601, Abcam, UK)
- Coplin jars
- Water bath
- Silanized slides (Sigma-Aldrich)

Required solutions

- Phosphate buffer solution (PBS) (1 sachet in 1 L deionized water, pH 7.4)
- 1% aqueous potassium permanganate solution (Univar, Redmond WA, USA): 10 g in 1 L distilled water
- 2% aqueous oxalic acid solution (Univar): 20 g in 1 L of distilled water
- 0.1 M Tris buffered saline (TBS): 12.1 g Trizma base, 9 g sodium chloride in 1 L distilled water (adjust pH 7.6 using HCl/NaOH)
- Nickel-diaminobenzidine solution: Mix 2% Nickel (II) sulphate in 0.1MTris buffer (TBS), adjust pH to 7.4 using NaOH/HCl. To this add DAB 0.5 mg/ml and mixed well and filtered through 0.2 µm syringe filter before use. If intensification by Nickel (II) sulphate is not desired then add DAB 0.5mg per ml of 0.1MTris buffer (TBS) only.
- Ethanol solutions at a concentration of 50%, 70%, 80%, 90, 95% and 100%
- Normal saline 0.9% (9 g NaCl/L in distilled water)

Tissue collection and preparation

Tissue samples (conjunctiva, skin samples with intact feather follicles and feather tips) were collected at days 5 and 9 post-infection from 30 2 to 3 week old meat (White Ross, $n = 15$) and layer (ISA Brown, $n = 15$) chickens as part of another study (Nazir et al., unpublished). Birds were occularly inoculated with ILTV Australian field strains (classes 9, 10 and 14) at a dose of 10^4 $TCID_{50}$/bird (University of New England Animal Ethics Committee, approval no. AEC18–040). An additional five birds were inoculated with sterile cell culture medium and served as controls. After fixation in 10% neutral buffered formalin for 48 h, the tissues were routinely embedded and sectioned at 5 µm on silanized slides. A control slide was also prepared which was not subjected to melanin bleaching.

Antigen retrieval method

1. Preheat slides at 60 °C for 1 h in an incubator and fill heat resistant coplin jars with modified citrate buffer solution to cover slides and then equilibrate in 90 °C in water bath
2. Deparaffinise slides in xylenes using two changes of xylene for 5 min each and then hydrate sections gradually through graded ratios of ethanol to distilled water. The authors suggest two changes of absolute ethanol, one change each of 90%, 80% and 50% ethanol and then distilled water for 5 min each.
3. Immerse slides in preheated modified citrate buffer solution, cover container with lid and incubate for 30 min after the set temperature has been achieved.
4. After 30 min remove the Coplin jar from the water bath and allow it to cool for 20 min at room temperature.
5. Rinse slides with tap water by gently introducing by small flow of tap water into the coplin jar.

Melanin bleaching procedure

6. Drain tap water from slides and immerse slides into a 1% aqueous potassium permanganate solution (oxidising agent) for 5 min.
7. Take slides out of the above solution and immerse immediately in 2% aqueous oxalic acid solution (decolourising agent) for 1–2 min, or until the brown colour on the sections completely disappears.
8. Wash the slides in running tap water for 2 min by following step 5 above, followed by gently dipping in distilled water.

Immunohistochemical staining

9. These steps are performed in a moist slide chamber. The easiest way to prepare this is to soak absorbent paper in distilled water and then place these in the bottom of the slide box. Slides can then be placed horizontally without significant loss of incubating solution. Encircle each tissue section on the slides with a PAP pen to reduce cross reactivity and then block endogenous peroxidase activity on the sections by incubating for 10 min in 3% hydrogen peroxide (5 ml of 30% hydrogen peroxide in 45 ml of PBS) follow this step by gently irrigating these slides TBS with PBS at room temperature
10. Prepare blocking solution of 10% equine serum and 0.1% Triton in PBS
11. Incubate slides with the blocking solution for 1 h at room temperature.
12. Tip blocking solution off the slides and then incubate with ILTV glycoprotein E polyclonal antibody diluted 1:200 in PBS overnight at 4 °C in the moist chamber. Note: In conjunctiva sections, incubation overnight at 4 °C is preferable to 1 h incubation at room temperature as it increased the positive staining intensity.
13. After washing in PBS, incubate slides for 2 h (room temperature) with anti-rabbit HRP diluted 1:500 in PBS.
14. Divide the Nickel-DAB solution into two coplin jars. Tip antibody solution off slides and then immerse slides in the first jar for 5 min and then in the second jar (for 5 min) to which 0.012% hydrogen peroxide (4 µl of 30% hydrogen peroxide in 10 ml nickel-DAB solution) is added and mixed by swirling just before immersing the slides. Note. If enhancement of the DAB precipitate is not required, the nickel sulphate solution can be omitted.
15. In another coplin jar, immerse slides in 0.9% normal saline for 1 min and then counterstain with Harris haematoxylin by rapidly dipping slides in this stain in a fresh coplin jar. Rinse slides well with distilled water.
16. Dehydrate and clear the slides by immersing in increasing concentration of ethanol (the authors suggest 50%, 70%, 95%, 100% 5 min each). Follow this step by clearing slides through two changes of xylene for 5 min each.
17. Mount slides with DPX and dry for around 18 h before viewing.

Please note that each step of the immunostaining procedure described is preceded by three washes with 0.1 M TBS for five minutes each time.

Method validation

In the current study, melanin was successfully bleached from chicken feathers using potassium permanganate and oxalic acid prior to immunoperoxidase staining using DAB as the chromogen, as shown in Fig. 1. To determine if the current bleaching method affects the antigenicity of the ILTV antigens, we used the same bleaching protocol on known positive control samples (conjunctiva) and negative control (by replacing primary antibody with PBS in sections of same conjunctiva samples). No appreciable difference was observed in the intensity of immunostaining between these samples and the known positive conjunctiva samples showed clear positive staining in conjunctival epithelium

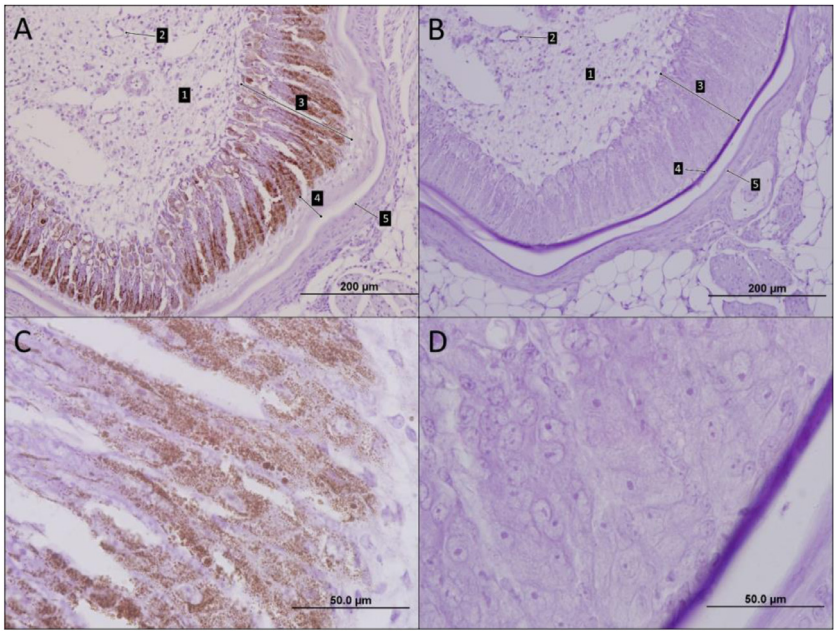

Fig. 1. Differences between unbleached (A, C) and melanin bleached (B, D) feather follicles in a ILTV negative sample. In Figs. 1A and B, tissue is shown at 20x magnification and major structures are highlighted (1 = feather pulp, 2 = axial blood vessel, 3 = barb region, 4 = feather sheath (some separation noted in B), 5 = feather follicle). Fig. 1A shows unbleached tissue, where large brown deposits of melanin can be seen within the barb region of the growing feather. Fig. 1B shows successful removal of these deposits by our method. Fig. 1C shows high magnification detail of the barb region and melanin granules are clearly noted (100x magnification), whilst Fig. 1D shows the removal of these granules and clear visualization of the underlying structures.

after bleaching (Fig. 2). The lack of difference in bleached and non-bleached conjunctiva tissues with no endogenous melanin confirms that the applied method of bleaching did not affect the antigenicity of ILTV glycoprotein E antigen. Similar findings were reported by Morris et al. [14] on *Renibacterium salmoninarum* bacterium in kidney tissues of rainbow trout. McGovern and Crocker [12] compared several methods of melanin bleaching on human normal skin and skin with malignant melanomas and concluded that the combination of potassium permanganate and oxalic acid was the method of choice. This technique is a simple, fast and practical method for bleaching chicken feathers, which can be safely applied to immunohistochemistry studies without any damage or loss of sections from slides and without any alteration of antigenicity. Bleaching should be performed after antigen retrieval as bleaching prior to antigen retrieval leads to detachment of sections during their incubation in antigen retrieval solution. Similar findings have been reported by McGovern and Crocker [12], Alexander et al. [1], Orchard and Calonje [17].

In this study, the frequency of feather follicles with high melanin was relatively low in white feathered chickens (6%, 1/15 White Ross broiler chickens) and moderately high in brown feathered chickens (40%, 6/15 ISA Brown layer chickens), which can be erroneously be interpreted as evidence of antigen detection. In fact, the development of this assay was motivated when 3/5 feather sections of negative control birds presented immunostaining. The presence of melanin in chicken feathers and the possibility of non-specific reaction when using DAB-based immunostaining have not been previously reported in previous studies investigating viral antigen in chicken feathers [10,11]. Therefore, we recommend a melanin removal step when DAB-based immunoperoxidase staining procedures are used in this sample type. This technique could also be beneficial for the detection of other pathogens in chicken feathers or in other melanin rich tissues in chickens such as the eye. However, the effect of bleaching on antigenicity could vary with the type of antigens [8] and therefore it is strongly

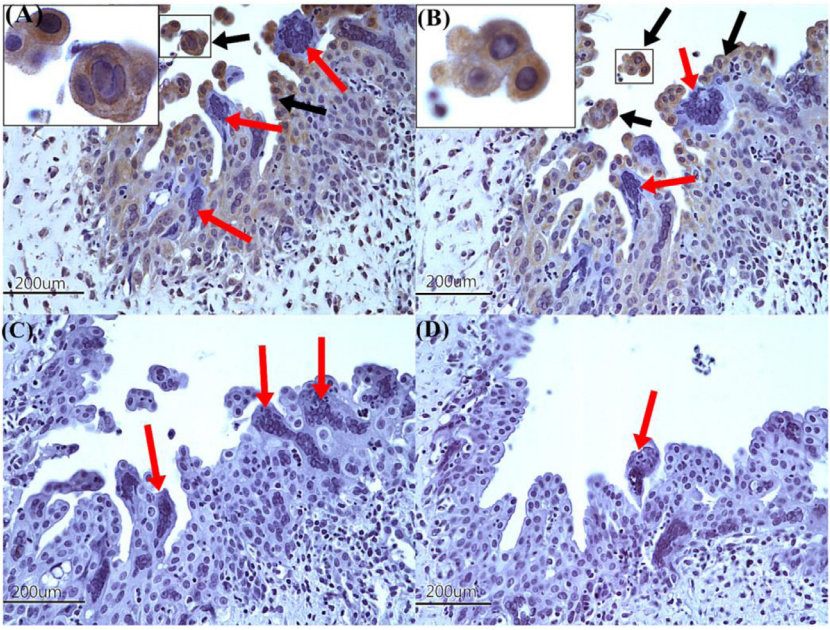

Fig. 2. Highlights staining of ILTV in a positive control sample (conjunctival epithelium) (Fig. 2A) and a negative control conjunctiva sample (where primary antibody was replaced by PBS)(Fig. 2C). Both positive and negative controls were treated with the melanin bleaching procedure described in this paper prior to antibody staining; Fig. 2A shows positive ILTV staining (black arrow) in conjunctival epithelium (positive control) at 40x magnification (without melanin bleaching steps). Fig. 2B shows no significant changes in ILTV staining (black arrow) in positive control conjunctival epithelium when melanin bleaching was done prior to antibody staining (40x). Fig. 2C and D shows no positive ILTV staining in conjunctival epithelium (negative control) when IHC was done with (Fig. 2D) or without melanin bleaching (2C) (magnification 40x). Syncytial cells (red arrow) and desquamation of epithelial cells also visible in Fig. 2A,B, C, D (also at high power 100x).

advised to test the effect of this bleaching method on the detection of other antigens during DAB-based immunoperoxidase staining procedures.

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Declaration of Competing Interest

There are no conflicts of interest

Additional information

Melanin pigment is produced by melanocytes that migrate into the epidermis during development. The pigment is mainly found in organs that receive light (skin and hair of mammals and feathers of

chickens) where they provide protection to cells from UV light. The pigment also plays a vital role in thermoregulation, camouflage, signalling and provide protection from abrasion, oxidative stress (act as a free radical scavenger) and pathogens [13]. In birds, the pigment makes the feathers denser and more resistant to wear and photochemical degradation [18].

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